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In Vitro Antigene Therapy Targeting HPV-16 E6 and E7 in Cervical Carcinoma

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Human papillomavirus (HPV) infection is believed to play a central role in cervical carcinogenesis. Specifically, two viral oncoproteins, E6 and E7, possess transforming ability and have been shown to interact with the cellular tumor suppressors p53 and p105, the retinoblastoma (Rb) gene product. To test the hypothesis that E6 and E7 play an active role in the maintenance of the malignant phenotype and may be ideal targets for antigene therapy, we tested the antiproliferative effects of phosphorothioate oligodeoxynucleotides (oligos) targeting HPV-16 E6 and E7 in cervical cancer cell lines and primary tumor explants. The ATP cell viability assay was used to measure growth effects of 27-mer antisense oligos targeting the ATG translational start region of HPV-16 E6 and E7 sequences in HPV-16-positive cell lines SiHa and CaSki and four advanced, primary cervical tumor explants. A random oligo sequence, an HPV-18-positive and HPV-negative cell line, one histologically confirmed endometrial and two ovarian tumors were used as negative controls. HPV type was confirmed by hybrid capture techniques. Cell lines and sterile (staging laparotomy) tumor cells were plated at 5000 cells/0.1 ml and 100,000 cells/0.5 ml in 96-well plates or soft agar, respectively, and incubated at 37°C with a single treatment of oligos at 0–16 μM. E6/E7 combinations at a fixed ratio of 1:1 were used at 0–8 μM for each oligo. Cellular ATP was measured by luciferin/luciferase fluorescence on Day 6. HPV-16 E6 and E7 oligos showed antiproliferative effects in all HPV-16-positive cell lines and primary tumor explants (IC_{50}s 6.9–9.5 μM for cell lines, 9.1–12.1 μM primary cervical tumors), while the HPV-negative C33-A cell line and HPV-18-positive cell line HeLa were relatively insensitive to the HPV-16 oligos (IC_{50}s > 30 μM extrapolated). The endometrial and two ovarian primary tumors were also insensitive to the HPV E6 and E7 oligos (IC_{50}s > 25 μM extrapolated). Random oligos had little effect on cell growth at concentrations up to 16 μM (< 25% inhibition), except in CaSki (@50% inhibition at 16 μM). Combinations of E6 and E7 demonstrated mixed synergistic and antagonistic effects as determined by combination indices (CI) derived from median effect parameters. In the HPV-16-positive primary cervical tumors and the cell line SiHa, E6/E7 combinations were synergistic at low doses (<25% growth inhibitory dose range) and antagonistic at doses above this. For the HPV-16-positive cell line CaSki, however, E6/E7 combinations were antagonistic at all dose ranges. Phosphorothioate oligos directed against the viral oncogenes E6 and E7 were shown to have antiproliferative effects specific to HPV-containing cancer cells. These specific antiproliferative effects suggest that HPV-16 E6 and E7 sequences play an active role in the malignant growth properties of cervical cancer cells and may be ideal targets for antigene therapy.

INTRODUCTION

Advances in the understanding of the role of HPV (human papillomavirus) in cervical cancer have produced strong evidence confirming the importance of the viral–host cell interactions in the etiology of cervical carcinoma. While a variety of other factors play a role in cervical carcinogenesis, 90% of squamous cervical carcinomas contain HPV genes that are believed to function as oncogenes [1]. If these viral oncogenes are actively involved in the maintenance of the malignant phenotype, then they are ideal targets for directed antigene therapy. Targeting genetic alterations unique to cancer cells may be clinically advantageous by selective inhibition of malignant cells while sparing normal cells. If, however, HPV initiates permanent alterations which subsequently lead to the development of malignancy, then targeting viral factors should produce little benefit. Expression of HPV oncogenes has been shown to be required for the establishment *and maintenance* of the malignant phenotype in human keratinocyte cell lines [2], but the fundamental question of the *active* role of HPV in human cervical cancers remains unclear.

We sought to directly test the hypothesis that HPV oncogenes play an active role in the maintenance of malignant

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growth properties. By targeting the expression of HPV-16 E6 and E7 genes with phosphorothioate oligodeoxynucleotides (oligos) in cervical cancer cells, growth effects were examined with the ATP cell viability assay. We have utilized both cell lines and primary cervical tumor explants to test this hypothesis.

Storey *et al.* [3] demonstrated that phosphorothioate oligodeoxynucleotides complementary to various E6 and E7 target sequences have specific and nonspecific inhibitory effects using micromolar concentrations of oligos. Interestingly, however, the rate of synthesis of E6 and E7 proteins and the steady-state levels of E7 mRNA were shown to remained largely unchanged. Steele *et al.* [4] showed that oligos targeting the start codons of E6 and E7 in HPV-18 containing cell lines produced specific antiproliferative effects. Tin and Tang published two articles relating to anti-E6 and E7 oligos in cervical cancer cell lines and in nude mice using bicistronic mRNA and phosphorothioate oligonucleotides, both studies demonstrating antiproliferative effects against the CaSki cell line [5, 6].

The rationale for targeting E6 and E7 stems from the evidence that these viral oncogenes interact directly with the cellular tumor suppressors p53 and p105/Rb (the retinoblastoma gene product) [7]. E6 has been shown to cause ubiquitin-mediated proteolysis of p53, while E7 binds to and inactivates Rb [7–9]. Previous studies have shown that the most effective oligos against E6 and E7 target the translational start sequences [3, 4]. Though translation inhibition and activation of cellular RNase H's are thought to be key mechanisms, the precise action of these antisense oligos has not been determined.

We examined the effects of phosphorothioate-modified oligodeoxynucleotides which are complimentary to viral mRNA (antisense oligos) specifically targeting the E6 and E7 ATG regions of HPV-16. Fresh cervical tumor explants were confirmed to contain HPV 16 as well as cervical epithelial tumor cell lines either containing (SiHa and CaSki) or lacking (C33-A and HeLa) HPV 16. C33-A contains no HPV DNA, while HeLa contains HPV-18 DNA [10–12]. In this study we have demonstrated that the antisense oligos targeting the translation initiation sites of either E6 or E7 inhibited proliferation of HPV 16-containing cells when compared to cells lacking HPV 16.

MATERIALS AND METHODS

Cell lines. SiHa, C33-A, HeLa, and CaSki were obtained from the American Type Culture Collection. Cell lines were maintained in Eagle's modified essential medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 g/ml amphotericin B in incubators at 37°C, 95% humidity, and 5% CO_2. Confluent cell monolayers were used for subculturing and to prepare cells for oligo treatment by detaching them with 0.25% trypsin/0.02% EDTA, manual counting with a hemacytometer, and dilution with media at 37°C.

Oligos. Phosphorothioate oligodeoxyribonucleotides 27 bases in length (M_r ~8700) were synthesized by an automated DNA synthesizer (Applied Biosystems 394 DNA/RNA Synthesizer) in 1 mM batches to 60–70% purity. Phosphorothioate oligodeoxyribonucleotides were synthesized with all phosphodiester positions modified. Oligo preparations were ethanol precipitated under sterile conditions, the pellets were resuspended in sterile water, and concentrations were determined by OD at 260 nm, with OD 260:280 nm ratios ranging between 1.5 and 2.0, consistent with single-strand oligos. Stock solutions of oligos were then prepared by dilution with the same medium used for cell incubations.

The antisense oligos for sequences and targets for E6 and E7, along with the scrambled control sequence (random sequence), are shown in Table 1 [13, 14]. A control sequence was generated from the corresponding antisense sequence of E6 using random number tables.

Treatment of cell lines. Cells were treated in 96-well microtiter plates by first diluting oligos in sterile media and then adding the counted cell suspensions to 5000 cells/well. Final oligo concentrations were 16, 8, 4, 2, and 1 μM in a final volume of 100 μl/well. For experiments using a combination of oligos, fixed 1:1 ratios of the two oligos were used at concentrations of 8, 4, 2, 1, and 0.5 μM each. All oligo concentrations were tested in triplicate for each experiment, and each experiment was repeated a minimum of three times. Incubations were carried out for 6 days without replacement of media or oligos.

Preparation of cervical tumor cells. Fresh cervical tumor specimens were obtained intraoperatively and transported to our laboratory for processing. The four primary cervical tumors were surgical IIB, two IIIB's, and two recurrences. All involved squamous histology, and tissues were obtained from gross nodal disease or parametrial breakthrough. No tissue from the vagina/cervix itself was obtained due to the possibility of bacterial contamination. A 1- to 2-cm piece of the fresh tumor tissue was set aside prior to processing the tumor cells and tested for HPV type using hybrid capture assays specific for HPV-16 (Hybrid Capture System, HPV 16 Probe Pack)[15]. Tumors not containing HPV-16 subtypes were excluded from oligo testing.

Briefly, the fresh tumor tissue was submersed for 20 min in Hanks' balanced salt solution (HBSS) containing penicillin and streptomycin. Connective tissue and fat are then grossly dissected to minimize nonmalignant cell content. The remaining tumor tissue is then finely minced and incubated overnight in HBSS containing 5% fetal bovine serum (FBS), 1× penicillin and streptomycin, 2.25 PUK/ml pronase, 0.2 mg/ml collagenase, and 1400 U/ml DNase. Disaggregated tissue fragments and cells are then washed twice in HBSS with 5% FBS and suspended in enriched Cannaught Medical

TABLE 1

The Phosphorothioate Oligodeoxyribonucleotide Sequences Used in This Study

Oligo	Sequence 1 3 5 7 9 11 13 15 17 19 21 23 25 27	G*C
		(%)
E6 antisense	5' A C A T T G C A G T T C T C T T T T G G T G C A T A A 3'	37
E7 antisense	5' T G T A T C T C C A T G C A T G A T T A C A G C T G G 3'	44
Random	5' G T C G A C T A A T C C G T T T T G A G A T A C T T T 3'	37
	• ••• •••••••••• • •••	% Mismatch
HPV 16 E6 consensus	5' T T A T G C A C C A A A A G A G A A C T G C A A T G T 3'	67%
HPV 18 E6 consensus	5' C T A T G G C G C G C T T T G A G G A T C C A A C A C 3'	
	•• •••• •• ••• •••	
HPV 16 E7 consensus	5' C C A G C T G T A A T C A T G C A T G G A G A T A C A 3'	52%
HPV 18 E7 concensus	5' A T A A T A T T A A G T A T G C A T G G A C C T A A G 3'	

Note. The anti-E6 sequence targets the E6 and E7 ATG regions of HPV 16 [18]. The sequence complimentary to the AUG translation start condon of the E6 transcript is underlined in bold. Asterisk denotes mismatch.

Research Laboratory (CMRL) media. Large tissue fragments and cell aggregates are allowed to settle for 2 min. followed by aspiration of the upper media layer, which contains both single cells and small cell clusters. Approximate viable cell counts are obtained using trypan blue dye exclusion. Cells were diluted to 200,000 viable cells/ml and aliquoted at 100,000 cells/500 μl in 24-well plates containing an agar underlayer to minimize nonmalignant cell growth. Use of agar underlayers has been shown to effectively abolish the growth of nonmalignant cells such as fibroblasts [16].

Treatment of primary tumor cells. Serial dilutions of 16, 8, 4, 2, and 1 μM of each oligo (E6 and E7), as well as Day 0 and 6 untreated controls, were plated out in triplicate on 24-well plates containing 100,000 cell/well with agar underlayers (total volume = 0.5 ml). Combinations of oligos at a 1:1 ratio were plated at concentrations of 8, 4, 2, 1, and 0.5 μM for each oligo. Incubations were carried out at 37°C for 6 days with no media changes or additional treatments.

ATP cell viability assay. On Day 6 of incubation, intracellular ATP was extracted from the cells in each well and quantitated by the ATP cell viability assay as previously described [17]. Cells were lysed by 1 vol of 4% trichloroacetic acid, and a 50-μl aliquot was transferred to a clear plastic tube and neutralized by 2 vol of 0.1 M Tris buffer, pH 9.2. A 20-μl aliquot of this was transferred to another clear plastic tube for luminometer reading. The luminometer (Packard Picolyte) injects 50 μl of Picozyme luciferin–luciferase complex into the tube and ATP-driven fluorescence is measured for 20 sec. Standard ATP solutions are diluted and assayed with each preparation of luciferin–luciferinase complex to establish a standard ATP curve confirming linearity over the experimental range of fluorescence detection (10^{-13} to 10^{-10} mole ATP/20 μl aliquot). Fluorescent units were normalized to untreated controls to determine percentage of control ATP.

Data analysis. IC_{50} was defined as the oligo concentration required for 50% growth reduction as compared to untreated controls and was calculated from the median effect analysis by the methods of Chou and Talalay [18]. This calculation is derived from the x intercept determined by linear regression analysis of the log (f_a/f_u) vs log(C) plot, where f_a is the fraction affected, f_u is the fraction unaffected $= 1 - f_a$, and C is the concentration. Correlation coefficients for individual ATP experiments averaged 0.957 ± 0.005 (SE), range 0.860–0.998. Since experiments from fresh primary tumors could not be repeated separately, primary tumor data were pooled for statistical purposes (see Table 2). All cell line experiments were repeated separately a minimum of three times.

Synergistic vs antagonistic effects were quantitated by combination indexes as calculated by the methods of Chou and Talalay [18], where at a given $f_a = x$, CI $= [(Dx)_1/(Dx)_1] + [(D)_2/(Dx)_2]$, with $D_{1,2}$ = experimental drug concentrations, and $(Dx)_{1,2}$ = calculated drug concentrations derived from the median effect parameters of the individual agents 1 and 2 at $f_{a=x}$. CI's less than, equal to, or greater than 1 represent synergism (supraadditive), summation (additive), or antagonism (subadditive) effects, respectively.

Statistics. Differences between IC_{50}'s of oligo–cell treatment groups were calculated by standard two-tailed t tests using Statview 4.1 on a Macintosh platform.

RESULTS

Table 2 shows the IC_{50}s ± standard errors for the cell lines and primary tumors for each of the oligos or oligo combinations tested. The IC_{50}s for the HPV-16-positive cell lines SiHa and CaSki were about fivefold lower than the corresponding IC_{50}s for the cell lines HeLa and C33-A lacking HPV-16, indicating that the inhibitory effects were spe-

TABLE 2
IC_{50} ± Standard Errors (μM) Values for Single and Combination Oligo Treatments in the Four Cell Lines Tested and the Cervical Tumors

	IC_{50} ± SE							
	E6	SE	E7	SE	E6 + E7	SE	Random	SE
Cell lines								
HPV 16(+)								
SiHa	8.72	1.12	6.70	0.49	8.38	0.96	27.94	4.28
CaSki	9.07	0.70	9.48	1.62	9.93	0.78	16.10	11.24
Ave	8.90	0.18	8.09	1.39	9.16	0.77	22.02	5.92
HPV 16 (−)								
HeLa	42.50	22.04	30.20	16.50	14.93	2.60	40.06	22.09
C33-A	67.95	22.25	128.35	87.94	71.49	22.96	130.43	49.66
Ave	55.23	12.73	79.28	49.08	43.21	28.28	85.25	45.19
Primary tumors								
HPV 16(+)								
Cx 1148	8.83		5.49		9.86		61.53	[illegible]
Cx 1055	12.51		7.10		11.38		240.64	
Cx 1058	19.92		13.65		8.75		279.72	
Cx 1020	6.97		9.98		13.31		249.17	
Ave	12.06	2.86	9.05	1.79	10.82	0.99	207.76	49.46
HPV 16(−)								
Endometrial	>16		>16		36.49		>16	
Ovarian 1	26.39		26.86		458.71		62.04	
Ovarian 2	23.47		111.68		147.94		439.67	
Ave	>16		>16		92.21		>16	

Note. >16 = no dose response. IC_{50} could not be calculated from median effect parameters. The extrapolated IC_{50} values in all control (randomized oligo) treated cell lines fell beyond the experimental range of concentrations used (16 μM). IC_{50} values could not be calculated for the endometrial cell line used as control.

cific to HPV-16 homologous sequences. Random oligos produced minimal inhibition in all cell lines and primary tumors (<25% at the highest dose of 16 μM), except in CaSki, where 50% inhibition occurred at 16 μM. Calculated IC_{50}s for random oligos were significantly higher ($P < 0.05$, t test) than the E6 or E7 oligos in HPV-16-positive cells, except in CaSki ($0.06 < P < 0.18$, t test).

Anti-E6 oligos were significantly more effective than E7 in SiHa ($P = 0.04$, t test), but not in CaSki. In primary tumors, E7 oligos were generally more effective than E6, but this trend did not reach statistical significance (see Table 2). The average IC_{50}s of each oligo treatment group tended to be higher for primary tumors than cell lines. Direct comparisons between cell lines and primary tumor explants, however, are not useful given the different cell preparation and incubation conditions (see Materials and Methods).

Figure 1 shows the dose–response plots for each of the oligo treatments. As seen in Fig. 1a, the dose responses for E6 oligos targeting the HPV-16 ATG sequences cluster into two groups. The cell lines lacking HPV (C33-A) or containing HPV-18 DNA (HeLa) have flat dose responses similar to those of the random oligo controls (Fig. 1d), indicating that the absence of specific target sequences renders cell lines relatively resistant to the effects of these oligos. The HPV-16-containing cell lines SiHa and CaSki, on the other hand, demonstrate a steady dose response with >50% inhibition beginning around 8 μM oligos.

The dose–response plots for E7 demonstrate a similar clustering of curves (Fig. 1b). While the HPV-16-negative cell lines remained above 50% inhibition with relatively flat dose responses, the HPV-16-containing cell lines demonstrated steeper dose responses and >50% inhibition in the higher 8–16 μM dose range. The combination of E6 and E7, as expected, also produced two types of dose–response curves, although the separation of curves was less pronounced (Fig. 1c). The random oligos produced almost flat dose responses for all cell lines except CaSki.

To further extend our findings with cell line responses to HPV-16 oligos, four HPV-16-positive primary cervical carcinomas were treated with the same panel of HPV-16 oligos. One endometrial and two ovarian carcinomas were grown and treated in an identical fashion to serve as HPV-16-negative controls. Figure 2 again shows grouping of dose responses among the HPV-16 tumors and negative controls. While the HPV-16-containing primary tumors experienced >50% inhibition at doses of 16 μM, the non-cervical tumor controls and random oligo-treated cervical primaries demonstrated minimal growth inhibition at similar concentrations.

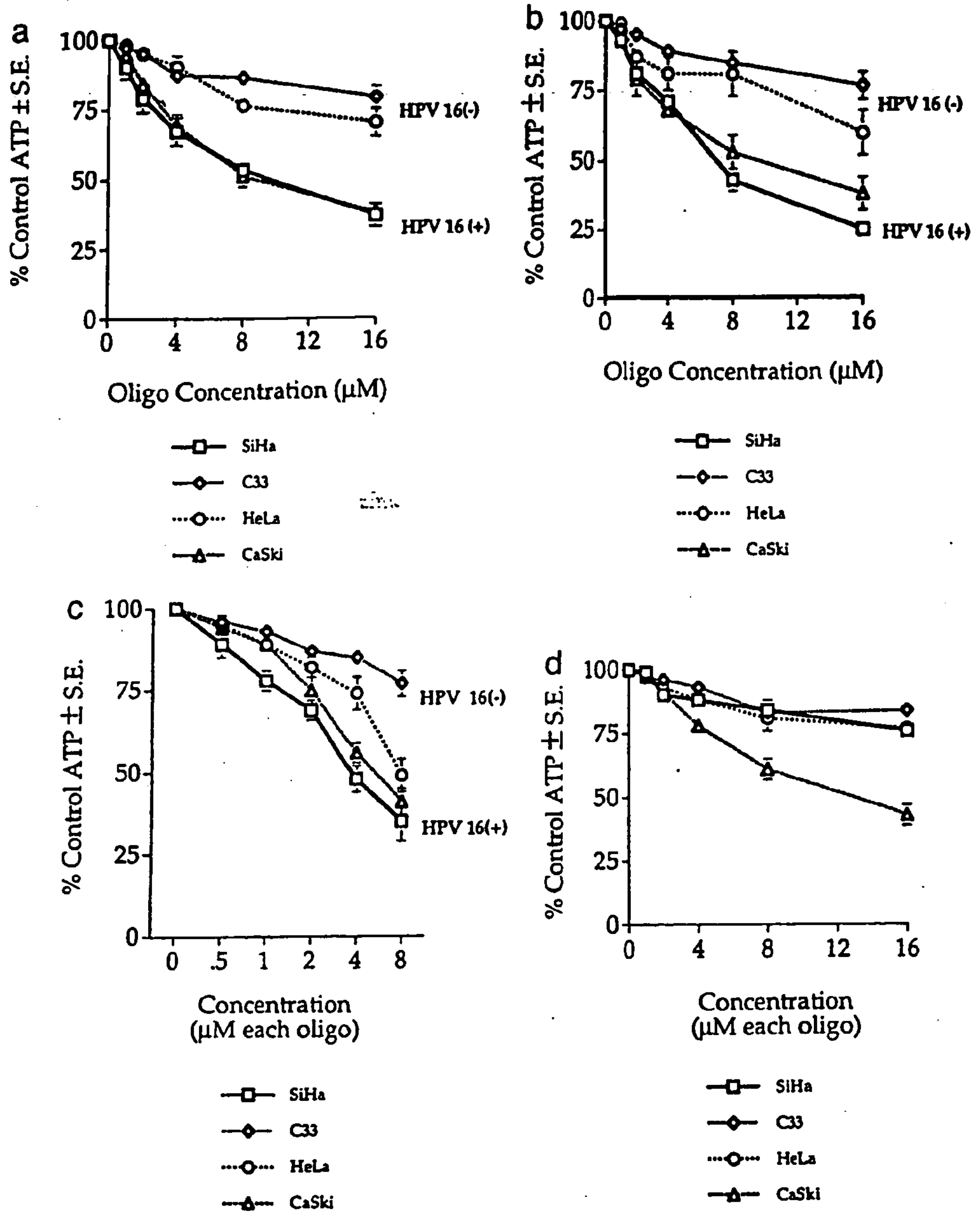

FIG. 1. (a) The reponse of the HPV 16(+) cell lines SiHa and CaSki contrasted with the HPV 16(−) cell lines HeLa and C33 to anti-E6 oligos. Responses clustered into two groups: HPV 16(−) cell lines demonstrating relative resistance to oligos, and HPV 16(+) cell lines exhibiting a steady dose response with >50% inhibition starting at 8 *μM*. (b) The response of HPV 16(+) cell lines SiHa and CaSki, and HPV 16(−) cell lines HeLa and C-33, to anti-E7 oligos at various concentrations.HPV 16(+) cell lines SiHa and CaSki containing the E6-targeted sequence experienced >50% inhibition at oligo doses >8 *μM*. HPV 16(−) cell lines C33 and HeLa remained above 50% inhibition demonstrating minimal oligo effects. (c) The response of HPV 16(+) cell lines SiHa, C33 and HPV 16(−) cell lines to equimolar combinations of anti-E6 and anti-E7 oligos. The dose–response curves for the HPV 16(+) cell lines exhibited inhibition even at very low doses (0.5 *μM*) with increasing levels of inhibition at higher doses. (d) The response of HPV 16(+) cell lines SiHa and CaSki and HPV 16(−) cell lines C33 and HeLa, to a randomized (scrambled) control oligo with similar G•C content to anti-E6 and anti-E7 oligos (see Table 1). Little effect was seen up to 8 *μM* oligo concentrations in all cell lines except CaSki, whose dose–response curve demonstrated increasing inhibition with increasing oligo concentration.

Thus the apparent HPV-16 specificity of HPV-16 E6 and E7 oligos applies to established cell lines as well as fresh cervical carcinoma cells in transient cultures.

To further examine the effects of combined targeting of E6 and E7, CIs were calculated from the median effect parameters as described under Materials and Methods. Con-

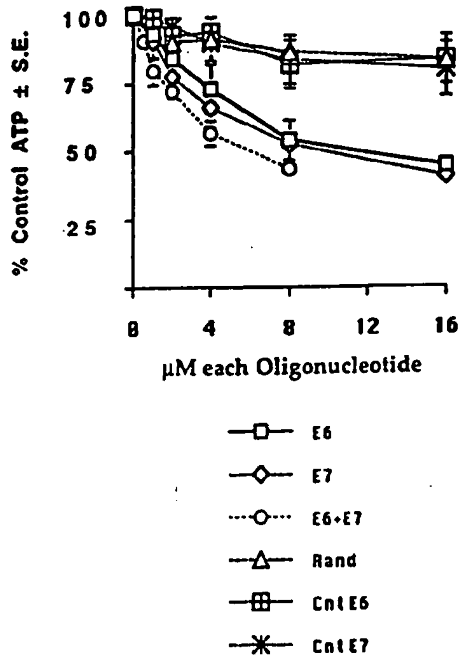

FIG. 2. The response of primary cervical tumors and control endometrial and ovarian tumors to anti-E6 and anti-E7 oligos. Confirmed HPV 16(+) cervical tumors were treated along with three HPV 16(−) controls (two ovarian tumors and an endometrial). HPV 16(+) cervical tumors experienced >50% inhibition at doses of 16 μM, while HPV 16(−) controls show minimal inhibition at similar concentrations.

trary to our initial expectations, E6 and E7 combinations resulted in predominantly antagonistic effects (Fig. 3). Antagonistic effects, however, were either minimal (CaSki) or more pronounced at concentrations of oligos *beyond* the experimental range calculated by median effect parameters. Synergistic effects *did* occur within the experimental range (fraction affected <50%, Fig. 3), but the significance of the *extrapolated* antagonistic effects is unknown.

DISCUSSION

We have previously shown that targeted antigene therapy against c-*myc* and p53 in ovarian cancer cell lines can result in antiproliferative growth effects [19]. Specifically, we have also found that oligos targeting p53 overexpression produce moderate reductions in detectable p53 expression and even more pronounced antiproliferative effects [20]. Interestingly, Storey *et al.* [3] have reported an analogous experience in which oligos targeting HPV-16 ATG start codon sequences produce specific (and nonspecific) growth inhibition in cervical cancer cell lines, even though the rate of synthesis of E6 and E7 proteins and the steady-state levels of E7 remained largely unchanged! They postulated that while specific effects of oligos targeting HPV sequences could readily be demonstrated, the relative absence of changes in protein levels suggests that these oligos may be acting through other, indirect mechanisms, perhaps via interaction with homologous cellular targets. Phosphorothioate oligos have been known to produce nonspecific inhibitory growth effects, predominantly through the nonspecific inhibition of polymerases [21].

While we have not examined alterations in gene product levels in response to E6 and E7 oligos targeting the ATG start codon, we have confirmed the basic findings of Storey *et al.* [3] and Steele *et al.* [4] that antiproliferative effects of E6 and E7 oligos could be tailored to HPV-containing cells. This provides evidence that E6 and E7 sequences play an active role in the maintenance of the malignant growth phenotype and that targeting these sequences may be the basis for future antigene therapies for cervical cancer.

Our data differ from those of Storey *et al.* in two respects. First, they observed inhibitory dose responses to E6 and E7 oligos in the HPV-negative cell line C33 (we did not). Second, their random control oligo produced little effect in the HPV-16-positive cell line CaSki, while we found that this was the only cell line that we tested which produced an inhibitory dose response to a random oligo (albeit moderate). Storey *et al.* speculated that their nonspecific effects might

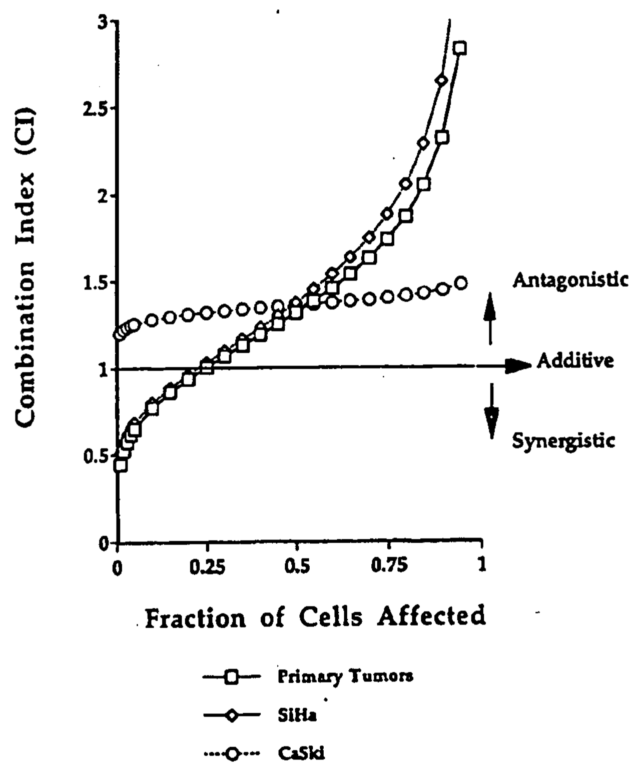

FIG. 3. Combination indices (CIs) calculated from the median effect parameters. Combination of anti-E6 and anti-E7 oligos resulted in predominantly antagonistic effects (CI > 1) at higher doses, while synergism was observed at lower doses (CI < 1).

be due to interaction with the human somatic cytochrome c gene and the c-*jun* protooncogene, which were 78–84% homologous with the HPV oligos [3]. While this possibility is intriguing, however, others have demonstrated that oligos experience a marked degradation in binding ability with even a single base pair mismatch [5]. In contrast to the paper by Tan and Ting, we have not found any significant difference between effects of E6 and E7 in HPV 16-containing cervical cancer cell lines [5, 6]. In fact, with primary cervical tumors, we have observed that anti-E6 oligos have a consistently greater inhibitory effect than anti-E7 oligos. We do not know the reason for this difference in findings, especially in view of the unpredictable oligo responses observed by various other researchers. Perhaps differences in methodologies play an important role in evaluating and comparing the effects of antigene oligos.

Cell line experiments were originally designed with the known HPV 16-negative HeLa and C33-A cell lines. In extending our investigation to primary cervical cell lines, we encountered a series of HPV-16-positive cancers without any evaluable (i.e., uncontaminated, viable in tissue culture) HPV-18 tumors. Analogous to C33-A, we therefore chose to use ovarian and endometrial controls to demonstrate the absence of response in HPV-negative tumors. Due to the prospective nature of acquiring primary advanced cervical tumors, however, we were not able to control the acquisition of HPV-18 primary tumors during the course of this investigation. Early-stage cervical tumors were not studied because of the technical inability in these tumors' tissue cultures (i.e., bacterial contamination). Early-stage cervical cancers tissue samples are usually obtained from septic biopsy specimens usually heavily contaminated, therefore making sterile tumor tissue cultures practically impossible.

The rapid ability to synthesize and test oligos targeting specific gene targets makes them exciting research (and potentially clinical) tools. A number of paradoxes and unexplained phenomena permeate the existing literature, however, and we have attempted to confirm and extend the antigene prospects for oligos targeting HPV sequences. We have generally confirmed the specific activity of these oligos in HPV-16-positive cells, and extended these findings to primary cervical explants derived from advanced, metastatic lesions. Although we have previously found combination antigene therapy to yield synergistic effects [19], combined targeting of E6 and E7 in this study unexpectedly produced mixed synergistic and antagonistic effects. Although this finding is difficult to explain, one possible mechanisms may involve interference of one oligo by the other, such as through nonspecific binding. The nucleotides 18–22 of anti-E6 oligos may bind to nucleotides 6–10 of anti-E7 oligos, thereby causing such an effect. Even weak or nonspecific binding of one oligo to another may decrease the antiproliferative effects to the point of causing an antagonistic net effect of the combination E6/E7 oligos.

Before the application of antigene oligo technology to the clinical setting for cervical cancer therapy, further *in vitro* studies are needed to determine the reliability and feasibility of this approach.

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